

COMMENTS ON THE VALUE OF COI FOR FAMILY-LEVEL FRESHWATER MUSSEL SYSTEMATICS: A REPLY TO HOEH, BOGAN, HEARD & CHAPMAN

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INTRODUCTION

Hoeh, Bogan, Heard & Chapman (2009; hereafter HBHC) raise a number of important criticisms of our recent analysis of family-level relationships among the lineages of the Palaeoheterodonta, Graf & Cummings (2006; hereafter G&C). We are pleased to have our conclusions scrutinized from other perspectives. HBHC also raise a number of questions about issues that we apparently did not explain in sufficient detail, and they report that the results of their reanalyses challenge our conclusions. We welcome their reanalysis and discussion. The objective of this article is to keep that debate alive by replying to HBHC.

HBHC discuss at least five points of disagreement with our methods of analysis or conclusions:

- (1) We did not include (or adequately justify exclusion of) COI sequences from the non-palaeoheterodont outgroup taxa (i.e., *Mytilus* and *Astarte*);
- (2) We did not include (or adequately justify exclusion of) COI sequences from three ingroup taxa (i.e., *Pseudomulleria*, *Coelatura*, and *Obliquaria*);
- (3) We did not transform our COI matrix to account for saturation;
- (4) We reached an erroneous conclusion about the monophyly of the Etheriidae; and
- (5) We reached an erroneous conclusion about the position of the Hyriidae.

These, of course, are all points open for debate, and we are grateful to HBHC for initiating this opportunity to consider them further. Their discussion of our methods and conclusions articulates well the differences in perspective we have regarding the ultimate purpose of performing phylogenetic analyses. We will take up each of these five points in order.

WE DID NOT INCLUDE COI FOR THE OUTGROUP TAXA

HBHC noted that we did not include COI sequences for either *Mytilus* or *Astarte* (the two non-palaeoheterodont outgroup taxa). As we explained in G&C, we excluded those sequences because they compromised the basal branches of our family-level phylogeny. This is evident in the reanalysis of the untransformed G&C matrix performed by HBHC (their fig. 5), as compared to G&C: fig. 2 (lower left: Combined Evidence, Problematic Included).

They correctly point out that if we had transformed our COI data, those effects on the basal branches of the phylogeny would have been minimized. Our general discussion of data transformation follows below. Our conclusion after preliminary analyses was that such divergent outgroup COI sequences contributed only confusion to the dataset and that their inclusion was unnecessary. With the exception of Graf & Ó Foighil (2000), previous analyses of the COI data applied in the G&C matrix did not include any non-palaeoheterodont sequences (Bogan & Hoeh, 2000; Hoeh et al., 2001, 2002; Roe & Hoeh, 2003). Rather, it had been assumed that the Palaeoheterodonta was monophyletic, and only *Neotrigonia* was necessary to root the unionoid tree. This is a reasonable assumption, given how well supported palaeoheterodont monophyly has been in previous studies of bivalve relationships (Hoeh et al., 1998; Giribet & Wheeler, 2002). We, however, wanted to include representatives from the pteriomorph and heterodont lineages in order to (at least nominally) test our hypotheses of the morphological synapomorphies diagnosing the higher-level taxa of the Palaeoheterodonta – something that had not been done before in the context of an analysis with representatives of all seven families of the subclass.

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As evidenced by every published COI analysis of freshwater mussel family-level phylogeny, this particular mtDNA fragment, while providing acceptable resolution within families, provides poor support for interfamilial relationships. Therefore, phylogenies derived solely from COI provide inadequate tests of hypotheses of freshwater mussel relationships at that level. Despite the fact that COI was a poor choice of characters for resolving those interfamilial relationships, it was the only molecular data available to a large sector of the tree (e.g., lasidium-bearing mussels) and had been used extensively in recent publications to make inferences about family-level relationships (Bogan & Hoeh, 2000; Graf & Ó Foighil, 2000; Hoeh et al., 2001, 2002; Roe & Hoeh, 2003). To meet the objectives of the G&C article to review and reanalyze the available data supporting the family-level phylogeny of the Unionoida, we had no choice but to rely on COI.

The problem with our untransformed COI matrix is that, even without *Mytilus* and *Astarte*, the data were full of noise – to the point that the majority of transformations traced to terminal branches (overall 56.9%, 1st position 56.9%, 2nd 62.0%, 3rd 56.8%; G&C: table 4). That is, most of the data were at best uninformative under parsimony, and given the amount of homoplasy observed (COI partition CI = 0.248; G&C: table 4), a large proportion were misinformative. We fail to see how this problem could be improved by adding two more long terminal branches to the analysis.

Transforming our matrix by excluding transitions occurring in the third codon positions would indeed have reduced the effects of saturation on the deeper nodes, but it would have also removed from the dataset many of the characters supporting shallower nodes (Swofford et al., 1996). Instead, we opted to limit the effects of COI to where it could do the most good or where it was the only sequence data available: the ingroup. In addition, we combined this faster-evolving mitochondrial gene with a 473 aligned-nucleotide fragment of the more-conserved nuclear locus 28S (where the data were available) as well as 59 morphological characters.

The fact that including *Mytilus* and *Astarte* COI sequences changes the ingroup topology is not an affirmation of the suitability of these data to the problem. Rather, it is indicative of the conflict within the dataset. G&C and HBHC each provided different methods for dealing with the problem of noise in the COI

dataset (both involving discarding portions of the available data because they were deemed inappropriate), but neither solved it – again, as evidenced by the weak interfamilial support in all analyses relying upon COI.

In summary, we did not include COI for the two outgroup species because doing so would have contributed only more noise to an already muddled dataset and because we had confidence in previous analyses of palaeoheterodont monophyly (Hoeh et al., 1998; Giribet & Wheeler, 2002).

WE DID NOT INCLUDE THREE PROBLEMATIC INGROUP COI SEQUENCES

HBHC are absolutely correct to be skeptical of a phylogenetic study that eliminates available data without justification. But, as we stated in G&C (p. 349), we excluded COI sequences for three taxa because of their “... inconsistency (relative to other available data) and their importance to family-level hypotheses of monophyly.” In the G&C paper, we ran all analyses both with and without the problematic taxa, provided a verbal description of the results on p. 353, and provided consensus cladograms (G&C: fig. 2). Moreover, on p. 374 we gave the following discussion:

“What makes these two sequences [*Pseudomulleria dalyi* and *Coelatura aegyptiaca*] problematic is not their conflict with the traditional classification. Rather, it is their inconsistent behaviour. In our COI Only analysis (not shown), these two sequences were placed in the strict consensus in a polytomy at the base of a (Unionidae + Margaritiferidae) clade that had no bootstrap support; Bogan & Hoeh (2000: fig. 1) resolved *Pseudomulleria* and *Coelatura* as the basal members of this clade. With the addition of 28S and morphology, *Pseudomulleria* migrated to a nonsensical position sister to the (*Unio* + *Cafferia*) clade, and *Coelatura* fell to near the base of the Palaeoheterodonta – and in at least a small number of MP trees, it was even placed outside of that clade (Fig. 2). When these two problematic sequences were excluded from the [Combined Evidence] analysis, *Coelatura* and *Pseudomulleria* were recovered in their traditional families, the Unionidae and Etheriidae, respectively (Figs. 2, 3). Scientifically, we are in no position to say that any of these various results is ‘incorrect’. However, it is our opinion that the available evidence does not warrant these two DNA se-

quences rejecting the null hypotheses that the Etheriidae and Unionidae are monophyletic.”

Obliquaria was discussed on p. 375. None of the concerns that we raised are addressed by HBHC. They simply reiterate their previous conviction that these inconsistently behaving sequences require no additional scrutiny.

Thus, we relied upon a dataset (and resultant phylogeny) that did not depend upon those problematic COI sequences because we had little confidence in the accuracy of the results those sequences provided in our analyses. The specific problem of *Pseudomulleria* is discussed below.

WE DID NOT TRANSFORM THE COI MATRIX

Regardless of whether or not HBHC refer to their parsimony analyses as unweighted, they are weighted since their process of matrix “transformation” is equivalent to an *a priori* weighting scheme. The matrix transformation to which HBHC refer has not, to our knowledge, been explicitly explained in previous papers, but we assume it refers to what Swofford et al. (1996) called “transversion parsimony.” For all third codon positions in the COI alignment, nucleotides are recoded as simply purines (G, A) or pyrimidines (C, T). This has the same effect as assuming a step-matrix wherein third position transitions are down-weighted to zero in the analysis of an untransformed matrix. When HBHC use the term “equal weighting,” they are strictly referring to how PAUP is treating their matrix: all character state changes in their “transformed” and “untransformed” matrices contribute equally to the final tree scores. However, by performing the transformation, HBHC have implicitly built their step-matrix into their character set rather than implementing a separate assumption set.

HBHC object to our not down-weighting the third position transitions of the G&C matrix in order to correct for the well-known problem of saturation in COI. In our analyses, we considered it sufficient to combine those COI data with a more conservative nuclear gene fragment (473 aligned positions of 28S) as well as 59 morphological characters. Thus, we added both more characters and more taxa to the troubled COI dataset, as has long been prescribed as a solution for dealing with long-branch issues and for minimizing the potentially misleading biases of individual data partitions (Graybeal, 1998; Hillis, 1998; Naylor & Brown, 1998).

MONOPHYLY OF THE ETHERIIDAE

As discussed by HBHC on p. 314, they disagree with our treatment of the Etheriidae as monophyletic. The G&C preferred dataset, which “... contained a *Pseudomulleria* terminal but only included morphological and life history characters in the matrix, confirmed the monophyly of the Etheriidae. Alternatively, Bogan & Hoeh’s (2000) analysis of COI DNA sequences, which included data for *Etheria*, *Acostaea*, and *Pseudomulleria*, indicates that *Pseudomulleria* is a unionid, as does Woodward’s (1898) anatomical study, thus rejecting the concept of a monophyletic Etheriidae.” Two aspects of HBHC’s argument require comment.

First, *Pseudomulleria* does not resemble a unionid in either its conchology or soft-anatomy. While it is true that Woodward (1898) used the word “Unionidae” to classify the two specimens of *Pseudomulleria dalyi* that he described and figured, he applied that term as an indication that this species was a freshwater mussel and not an oyster – that the anatomy was more similar to *Unio* and *Anodonta* than to other “... such monomyarian forms as *Meleagrina*, *Ostrea*, *Anomia*, and *Pecten*...” (p. 90). Further, Woodward considered both *P. dalyi* and *Acostaea rivoli* (= *lobata*) to belong to the same genus (*Mulleria*), indicating his assessment that those species are closely related. Nomenclatural argumentation aside, Woodward provided the following description of the mantle of *Pseudomulleria*:

“The mantle folds are almost completely separated from one another, being united at two points only, viz., at the posterior attachment of the gills, thus separating an inhalent from an exhalent orifice, and dorsally from the posterior end of the hinge-line for about one-quarter of the distance between this point and the attachment of the gills” (p. 87).

Whereas this description does not match what is known for any species of the Unionidae (e.g., there is no supra-anal aperture), the fusion of the mantle between the incurrent and excurrent apertures matches the condition otherwise known from only the Mycetopodidae and the Etheriidae. Most authorities of the 20th century that have studied the genera in question have noticed the striking conchological and soft-anatomical similarity between *Acostaea* (from South America) and *Pseudomulleria* (from India). Historical “Just-So” stories of convergent evolution in the characters of these lineages were invoked not because anyone thought

that the two monotypic genera were actually morphologically dissimilar but to explain their geographical disjunction in a paradigm lacking continental drift. This was commented upon by Yonge (1978: 440):

“Without realizing the effectively identical structure of the animals of *Acostaea* and *Pseudomulleria* or their unique manner of growth into the monomyarian adult, these two genera have been thought of as arising independently as a result of convergent evolution. ... But the changes both have undergone are so unique – so totally unlike those undergone by any other monomyarians – that it is excessively difficult to maintain that these could have arisen independently in two far distant river systems.”

That other freshwater mussels like *Modellnaia* (Heard & Hanning, 1978) and perhaps *Bartlettia* (Parodiz & Bonetto, 1963) arrived at somewhat etheriid-like conchologies through convergence does not automatically make asymmetrical shells resulting from a cementing lifestyle homoplastic in every case.

Secondly, HBHC, Bogan & Hoeh (2000), and subsequent works deriving conclusions from this hypothesized etheriid polyphyly (Bogan, 2007; Bogan & Roe, 2008) have given this single sequence of one gene fragment from one specimen a lot of weight. It has yet to be made explicit why this particular, oddly behaving (see above) sequence should be considered representative of *Pseudomulleria dalyi*. But more importantly, HBHC provide no evidence that they appreciate how incongruous what is known of the soft-anatomy of *Pseudomulleria* is with its placement among the unionids, and no specimen voucher or figure has ever been indicated. In the 10 years since the hypothesis was proposed, no additional data has been presented in support of etheriid polyphyly, and the rest of the data gathered over the past 110 years (meager as it is) stands against it.

In our study, we took a conservative approach and recognized that this single ambiguous data point was not sufficient to reject the traditional hypothesis of etheriid monophyly. Thus, we regarded this sequence as “problematic” and did not include it in our preferred matrix. (The same logic applies to the published COI sequences for *Coelatura* and *Obliquaria*, which we also deemed problematic and that bear upon the monophyly of the Unionidae and Lampsilini, respectively.)

RESOLUTION OF THE POSITION OF THE HYRIIDAE

Based upon the HBHC reanalyses of our G&C matrix, they find that only the unweighted parsimony analysis (their fig. 5) recovers a clade composed of (Hyriidae + Etheriidae + Iridinidae + Mycetopodidae), the same result reported from previous analyses emphasizing morphological characters. However, they prefer the results recovered by both Bayesian Inference (fig. 3) and MP analysis of their transformed matrix (fig. 4). The contentious position of the Hyriidae has been a persistent issue since the first phylogenetic analyses of freshwater mussels: morphology supports one result, COI another. Previous hypotheses of the phylogenetic position of the Hyriidae were discussed by G&C.

We reexamined for ourselves the COI portion of the G&C matrix (i.e., we excluded all taxa that had no COI sequences and all non-COI characters) under parsimony to evaluate further the power of COI to discriminate alternative topologies. All analyses were performed with the portable version of PAUP*4b10 (Swofford, 2002). Beginning with unweighted analyses, we performed three separate heuristic searches (“hsearch addseq=random nreps=100”): (1) unconstrained, (2) constraints enforced to recover only trees with a monophyletic Hyriidae basal to the rest of the species in the order (nicknamed “Hyriidae Basal”), and (3) constraints enforced to recover only trees with a clade composed of the species of the Hyriidae, Etheriidae, Mycetopodidae, and Iridinidae (nicknamed “Hyriidae + Etherioidea”). In the latter constraint tree, the *Pseudomulleria* sequence was allowed to fall among the (Unionidae + Margaritiferidae) species. Each of the constrained topologies was compared to a single unconstrained tree using both the Templeton and Winning-Sites tests (Felsenstein, 2003) (both implemented in PAUP*; “pscores /nonparamtest=yes”). We also performed the same tree searches assuming a step-matrix wherein third position transitions were down-weighted to zero (i.e., transformed). The results of these analyses are presented here in Table 1.

The Templeton and Winning-Sites tests are paired-sites tests that compare alternative topologies statistically, with p values indicating if one tree is significantly better than another, given the amount of conflict in the dataset (Felsenstein, 2003). Thus, a $p < 0.05$ indicates

TABLE 1. Results of nonparametric tests comparing unconstrained and constrained topologies using both unweighted and transformed COI data from G&C. A single tree unconstrained topology (“best”) was compared to the topologies recovered from searches with constraints enforced. Constraint “Hyriidae basal” = (Neotrigonia, (Hyriidae, (all other species))); constraint “Hyriidae + Etherioidea” = (Neotrigonia, (Hyriidae + Etherioidea), (all other species)). P-scores of Templeton and Winning-Sites nonparametric tests (Felsenstein, 2003) comparing each constraint topology with the “best” tree are given parenthetically. *indicates significance at the $p < 0.010$ level. No results were significant at the $p < 0.05$ level.

Topology	Unweighted	Transformed
Best	2307 (1 of 69 trees)	1044 (1 of 6 trees)
Hyriidae basal	2311 (0.6857, 1.0)	1044 (1.0, 1.0)
	2311 (0.7001, 0.9141)	1044 (1.0, 1.0)
		1044 (1.0, 1.0)
		1044 (1.0, 1.0)
		1044 (1.0, 1.0)
		1044 (1.0, 1.0)
Hyriidae + Etherioidea	2313 (0.5286, 0.9087)	1049 (0.3216, 0.3593)
	2313 (0.6599, 0.7545)	1049 (0.2513, 0.4545)
		1049 (0.2818, 0.3018)
		1049 (0.1967, 0.3877)
		1049 (0.0588*, 0.1250)
		1049 (0.1317, 0.2266)

that the null hypothesis that the data supports a constraint topology as well as the optimal topology is rejected at the 95% level. These tests are liberal, in that they can under-estimate p , providing false confidence in a particular optimal topology (Buckley et al., 2001). Nevertheless, in comparing the ability of COI to actually provide robust tests distinguishing alternative topologies, it performed poorly. Among the constraint trees tested, unweighted COI could not reject them, and transformed COI was able to distinguish only one of the six equally parsimonious “Hyriidae + Etherioidea” trees from the optimal topology (which in this case was one of the “Hyriidae Basal” trees) at the 90% level. Based upon these results and those of HBHC, transformed COI performs slightly better than unweighted COI, but neither performs well. This is reflected in the consistently low branch support for interfamilial relationships supported by COI (Graf & Ó Foighil, 2000; Bogan & Hoeh, 2000; Hoeh et al., 2001, 2002). We agree with HBHC that, when it comes to relying upon COI to recover such ancient divergences, “... we cannot objectively choose among interfamilial relationship hypotheses at this time” (p. 315).

However, COI is not the only dataset available to assess the most basal of freshwater mussel relationships, including the position of the Hyriidae. All morphological matrices analyzed to-date have provided the same result (when analyzed under parsimony): a sister relationship between the Hyriidae and the lasidium-bearing mussels (Graf, 2000; Hoeh et al., 2001; Roe & Hoeh, 2003). COI is equivocal on the subject of hyriid relationships, whereas morphology has been consistent in recovering a monophyletic Etherioidea that includes the Hyriidae – even when employing independently derived matrices.

The actual disagreement between G&C and HBHC in the case of the position of the Hyriidae (and perhaps the rest of the contentious branches on the freshwater mussel phylogeny) seems to be over whether or not the phylogenetic results to-date are robust enough to derive any conclusions, closing their discussion with (p. 315):

“Thus, robust estimates of unionoid bivalve (1) interfamilial relationships, (2) character evolution, and (3) lineage-specific species richness, as well as a stable, phylogeny-based

classification for the group, await appropriate phylogenetic analyses utilizing more inclusive datasets.”

We disagree: analyses to-date have been sufficiently robust to falsify a number of hypotheses, and even in the face of uncertainty and complexity, it is reasonable and useful to apply phylogenetic hypotheses as the tools that they are. These points get at the fundamental difference between our perspective and that argued by HBHC.

CONCLUSIONS

The goal of the G&C article was to put the classification of freshwater mussels on a cladistic foundation. Rather than being inductive and extrapolating generalities from the data, we set out to test hypotheses of the monophyly of the families, the interfamilial relationships, and the synapomorphies that support them. We used a combined evidence approach applying the available mtDNA, nuclear, and morphological datasets that had sufficient taxon sampling, and we drew upon the resulting phylogeny to meet our objectives. Moreover, our analyses were transparent – for example, we provided GENBANK accession numbers, museum vouchers, detailed descriptions of morphological characters, statistics of character-partition performance, and discussions of previous phylogenetic results. In addition, other unpublished materials (e.g., our complete matrix, results not shown) were made available on the MUSSEL Project Web Site – <http://www.mussel-project.net/>.

The G&C classification is a hypothesis to be tested. It has value only if it is useful as an organizing principle, a coherent evolutionary framework for freshwater mussel research. This is something that has been lacking despite more than a decade of cladistic analyses (Graf & Cummings, 2007) and something that the community has already put to good use (e.g., Barnhart et al., 2008; Rashleigh, 2008). But, the classification we proposed is just a hypothesis, and when it is shown to no longer be useful, it can be abandoned or modified. HBHC argue that COI is unreliable for testing interfamilial relationships of freshwater mussels. We agree, and that is why we did not rely solely on that limited set of characters. Moreover, G&C provided a conservative appraisal of the work to-date by not invoking ambiguous results to reject long-held hypotheses of relationship. HBHC have taken a bolder approach by introducing drastic

revisions to freshwater mussel classification, such as etheriid polyphyly and a novel subordinal classification (HBHC: fig. 2).

We regard the G&C classification of palaeo-heterodont families to be the best corroborated hypothesis of freshwater mussel relationships currently available. HBHC disagree. Based upon their concluding sentence (quoted above), it would seem that they are unsatisfied with the ability of any available phylogeny to distinguish among competing systems, although this is contradicted by their own proposed revisions. We expect that HBHC would agree with us that having a classification is preferable to none at all. Otherwise, if the plan is to suspend evolutionary considerations of freshwater mussel biology until the ultimate classification materializes *a posteriori* from the right (as yet unknown) combination of data and theory, we are afraid our efforts will have been wasted. Given the rapidly declining health of freshwater mussel populations around the world (Strayer, 2006) and our duty as systematists to provide a natural and useful classification for our stakeholders, the pressing problem of understanding the evolution and ecology of the Unionoida is too important to put off.

ACKNOWLEDGMENTS

The research that forms the basis of this discussion was funded by grants from the National Science Foundation. Various drafts were read and improved by Phil Harris and Diarmaid Ó Foighil, and our ideas were clarified through discussions with Kevin Roe. However, responsibility for all opinions expressed and any errors of fact belongs solely to the authors.

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Revised ms. accepted 24 September 2009